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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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**Blatt 2 der Bescheinigung  
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**Mass spectrometry-based technologies for continuous flow biossays using known ligands**

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Title: Mass spectrometry-based technologies for continuous flow bioassays using known ligands.

The present invention relates to the on-line coupling of mass spectrometry (MS) to continuous-flow separation techniques. In a further embodiment, this on-line detection method is used as either a scanning or monitoring method for assays, such as biochemical assays, using a known ligand. Furthermore, this invention relates to compounds detected by this method and the use of these compounds as a ligand for affinity molecules.

It is known from the prior art that biochemical assays are highly sensitive detection techniques which combine the selectivity of biospecific interactions with the sensitive detection of labels used as reporter molecules.

An example of such a technique is described in WO 91/13354. This document describes a flow immunosensor wherein an antibody specific to an affinity molecule is immobilized on a support. The antibody's binding sites are saturated with a labeled form of the affinity molecule. A liquid to be analyzed is contacted with the antibody to allow any affinity molecule present in the liquid to displace the labeled antigen. Finally, the displaced labeled antigen is detected.

However, it is known as well that immunoassays suffer from the problem of cross-reactivity, which means that antibodies react with more than one analyte. This leads to erroneous results. For this reason, immunoassays are frequently combined with a fractionation step, e.g., a separation step using HPLC or another type of liquid chromatography.

In the field to which the present invention relates, there is a demand for on-line coupling of a fractionation step and a biochemical assay detection system. Several approaches have been proposed and described to perform continuous-flow biochemical assays. Most of these immunoassays are in the form of a postcolumn reaction

This prior art assay is a heterogeneous detection system. It requires a separation step between free and bound label. Examples for free/bound label separation techniques are restricted-access phases and hollow-fibre modules.

5       The immunoreagent in the previously described prior art assay consists of fluorescein-labelled fragments of anti-digoxigenin antibodies which were immunopurified and are commercially available. The commercial availability of purified, labelled antibodies is however exceptional. In  
10 almost all cases, antibodies are only available in unlabelled state in crudely purified antiserum. Although labelling and purification schemes for antibodies (or other affinity proteins) are known to the person skilled in the art, it will be preferred to use antisera, which may be commercially  
15 available, without any pretreatment.

Furthermore, it is difficult to selectively label only those antibodies in an antibody preparation which react with the analytes. In general, labeling techniques make use of primary amine groups of antibodies resulting in the co-  
20 labeling of all other proteins present in the preparation. This leads to a drastic increase in the background signal and, consequently, to an increase in detection limits.

One aim of the invention is to provide alternatives for this known technique, viz. providing a technique which  
25 does not require the use of labeled antibodies. In addition, another aim of the present invention is to provide a technique that is not dependent on fluorescent behavior of labels, which makes it more versatile.

Hsieh et al. (Molecular Diversity, 2 (1996), 189-196)  
30 describe a screening method using chromatography coupled with mass spectrometry. Their method requires a dual run, first in the absence of a library of protein targets, and then with the library. This means that background compounds, especially the affinity proteins causing severe background signals over  
35 a broad range in the mass spectrum, are always present in

molecules react in a second step with an excess of a known ligand to titrate the remaining free binding sites; or as an alternative, a competition reaction occurs between the affinity molecule, the analyte in the effluent and the known  
5 ligand. Normally, the ligand/affinity molecule complex is detected after a separation of free and bound ligand, preferably on the basis of the difference in molecular weight. Finally, the ligand/affinity molecule complex is detected, either directly or after a dissociation step, with  
10 the MS.

The combination of the fractionation step with the MS detection step in accordance with the present invention greatly enhances the performance of both techniques. The combined techniques provide an analytical method which is  
15 characterized by a high selectivity and a high sensitivity. Further, the problem associated with cross-reactivity does not occur when using the method of the present invention. Furthermore, the assays with the method according to the present invention can be performed quantitatively.

20 The method of the present invention uses bioaffinity molecules such as antibodies, receptors etc. to detect any compounds showing high affinity for the ligand binding site of said affinity molecule. The compounds to be detected may be biochemical compounds but are not in any way restricted  
25 thereto.

The method of the invention makes it possible to screen a mixture of different compounds on their ability to bind to a certain known ligand, e.g., in order to find an inhibitor for said ligand. Therefore, the present invention  
30 further relates to a fast on-line method for the screening of compounds for their binding capability to a known ligand, which method comprises a fractionation step providing an effluent, the addition of a controlled amount of said affinity molecule to the effluent of the fractionation step  
35 and effecting a contact time sufficient to allow a reaction with or an interaction between the compounds in the effluent,

is subjected to detection using the affinity molecule detection technique; the other stream is directed to a fraction collector. Dependent on the signal obtained from the affinity molecule detector, fractions containing compounds causing a positive response will be collected while fractions causing a negative response will be discarded. This complete screening method can be automated using known valve-switching processes.

A suitable fractionation method to be used in the methods of the present invention comprises a liquid chromatography separation or a capillary electrophoresis step. Other separation or fractionation techniques which are known to the person skilled in the art and which allow a relatively continuous output stream can, however, be used as well.

In a preferred embodiment, the liquid chromatography separation step is a reversed phase HPLC step.

It will be understood that according to the present invention as the effluent of a fractionation step is also to be understood the effluent of a flow injection, in which a mixture of different compounds is injected, optionally followed by a trapping step, for example using a restricted-access column. The separation step of the method according to the present invention then fulfills the fractionation, since the compounds of interest are fractionated by the separation step. The required selectivity can be obtained by operating the MS selectively tracing of the MS.

Since the flow injection technique provides a flexible and fast screening method, it is a preferred embodiment of the present invention.

All modes of MS-operation are possible in Flow Injection mode, however, due to the higher background, especially the very selective modes which comprise detecting ions of a selected single  $m/z$  trace or of selected multiple  $m/z$  traces are suitable.

As already mentioned, the fractionation step can be a liquid chromatography separation, a capillary electrophoresis step or a combinatorial chemistry system. It has been found that it is vary advantageous when this fractionation step is followed by a separation step, for example by using a hollow-fiber module, since this removes the high molecular weight background. Preferred liquid chromatography fractionation steps comprise HPLC, reversed phase HPLC, capillary electrophoresis (CE), capillary electrochromatography (CEC), isoelectric focusing (IEF) or micellar electrokinetic chromatography (MEKC), all of which techniques are known to the person skilled in the art.

All possible variations in MS techniques known in the art can be profited from according to the present invention. preferably, the MS is of the type chosen from the group consisting of electrospray ionization type, atmospheric pressure ionization type, quadrupole type, magnetic sector type, time-off-flight type, MS/MS, MS<sup>n</sup>, FTMS type, ion trap type and combinations thereof.

For example, in scanning mode to trace compounds, low resolution MS with all possible instrumental designs of MS can be used, in particular quadrupole, magnetic sector, time-off-flight, FTMS and ion-trap. This generates typically molecular weight data with nominal mass accuracy.

When high resolution MS is applied, using all possible high resolution instrumental designs, in particular magnetic sector, time-off-flight, FTMS and ion trap, molecular weight data with high mass accuracy combined with the elemental composition of the compound can be obtained.

Other known MS techniques comprise tandem MS, such as MS/MS or MS<sup>n</sup> (for example MS<sup>3</sup>). Application of these techniques enables the collection of structural information of the ligands which is a preferred embodiment of the present invention. The data in scanning mode can be acquired in data-dependent mode which means that for each peak observed automatically the tandem MS measurement is performed. In

It is also possible to operate the MS in scanning mode. In this way the lowering of the pre-determined signal is correlated to the increase of other signals, enabling the active compound to be characterized in the same cycle.

5           With the assays according to the present invention the tracing of (bio-) active compounds in solutions of complex nature, for instance biological fluids or extracts, natural product extracts, solutions or extracts from biotechnological processes, resulting from chemical  
10 experiments, such as combinatorial technologies and processes and the like can be performed with a higher efficiency, selectivity and flexibility. Moreover, the present invention provides the possibility to limit the disturbance of background compounds.

15           The present invention further enables the identification of compounds based on conventional mass spectra, high resolution data or MS<sup>n</sup> based spectra.

          With the method of the present invention it is also possible to perform library searching, based on a variety of  
20 mass spectrometric experiments enabling the screening of large series of samples and classifying these in classes based on similar active compounds, without the need for full identification of the compounds.

          The assay method of the present invention can be  
25 applied in miniaturized formats of assay-based systems, for instance with chip technology based screening systems.

          In the methods of the present invention all molecules capable of interaction with or binding to other molecules can be used as affinity molecule. The affinity molecule can for  
30 example be selected from the group of cytosolic receptors, e.g., the estrogen, glucocorticoid receptors; solubilized membrane bound receptor, e.g., a  $\beta$ -receptor; affinity proteins, such as antibodies, enzymes, avidin; polynucleotides and polysaccharides.



26. 04. 1999

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Claims

1. On-line detection method comprising the on-line coupling of the effluent of a fractionation step to a mass spectrometer, which method comprises the addition of a controlled amount of an affinity molecule to an effluent of the fractionation step, whereby the affinity molecules bind analytes in the effluent, followed by the addition of a controlled amount of a known ligand capable of binding to the affinity molecule under suitable binding conditions, followed by a separation step to separate the free and bound known ligands and finally detection of either the free or bound known ligands using the mass spectrometer.
2. On-line detection method according to claim 1, in which the separation step comprises the retention of the free ligand from the effluent using a restricted-access support, whereby the ligand-affinity molecule complex is permeated, and the bound ligands are detected after being separated from said ligand-affinity molecule complex in a suitable dissociation step, followed by separation of the ligand from the affinity molecule using a hollow-fiber module, and directing the permeate stream containing the ligand to the mass spectrometer, in which method the dissociation step is preferably a low pH shock, contacting with a high ionic strength solution, contacting with an organic solvent and/or contacting with a chaotropic reagent.
3. On-line detection method according to claim 1, in which the separation step comprises the retention of the ligand-affinity molecule complex from the effluent using a hollow-fiber module, whereby the free ligand is permeated, and the permeate stream with the free ligand is subsequently directed to the mass spectrometer.

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Abstract

The present invention relates to the on-line coupling of mass spectrometry (MS) to continuous-flow separation techniques. In a further embodiment, this on-line detection method is used as either a scanning or monitoring method. Furthermore, this invention relates to compounds detected by this method and the use of these compounds as a ligand for affinity molecules.